Tibetan Plateau Relict Snakes of the Genus *Thermophis* and Their Relationship to New World Relict Snakes

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Abstract   The complete mitochondrial NADH dehydrogenase subunit 2 (ND2) gene sequences of two species of *Thermophis*, *T. baileyi* and *T. zhaoermii*, were sequenced and compared to those of 86 sequences from other snakes (74 from Caenophidia and 12 from Henophidia). By using Bayesian inference (BI) and maximum likehood (ML) approaches, *Thermophis* was demonstrated as the sister group to the North American relicts of Dipsadinae, and rooted in Central and South American members of this subfamily. The results suggest that the closest relatives of *Thermophis* are the North American relicts, and thus support the hypothesis for an Asian-North American origin of xenodontine snakes as suggested by Vidal *et al.* (2000). Extensive sampling of Asian snakes and American dipsadines is needed to further test this hypothesis in the future.

Keywords   *Thermophis*, Tibet, Dipsadinae, ND2, Asian-North American origin

1. Introduction

Hot-spring snakes are endemic to the Tibetan Plateau, known as "the roof of the world" and "the world's third pole". Their precise systematic position is still a question.

Initially, the hot-spring snakes such as *Tropidonofus baileyi* (= *Natrix baileyi*) from the Tibetan Plateau were placed within Natricinae (Wall, 1907). However, Malnate (1953) designated a new (monotypic) genus *Thermophis* within Xenodontinae for these snakes based on the characters from the hypapophysis and the hemipenis. Additionally, he considered *Thermophis* to closely resemble the ancestor of both the Natricinae and Colubrinae. *Thermophis* was placed in Colubridae *incertae sedis* on account of the hemipenial character of a divided sulcus in a single calyculate lobe (described by

Malnate, 1953) (Zaher, 1999). The molecular study of Lawson *et al.* (2005) retained this classification based on the previous studies.

Interspecific taxonomy within this genus and estimates of range did not change for nearly 100 years. *Thermophis baileyi* was thought to occur only in Lhasa and the Gyantze region (Malnate, 1953). However, *T. baileyi* also occurred in the middle reaches of the Yarlung Zangbo River such as in Lhatse and Chushur (Rao, 2000). Furthermore, 13 locations were recorded for discovering *T. baileyi* in the central part of the Tibet Plateau (Dorge *et al.*, 2007). *Thermophis* was also found in Litang County, Sichuan Province (Liu and Zhao, 2004). Most recently, after detailed morphological studies, the suggested populations from Litang were considered a new species and named as *T. zhaoermii* (Guo *et al.*, 2008). Huang *et al.* (2009) designated the hot-spring snakes (endemic to the Tibetan Plateau) as a "relict" species, according to Pinou *et al.* (2004). This designation was due in part to their unique ecological adaptations to the environment and climate of the Tibetan Plateau, but also to the lack of closely related taxa in Asia.

Using unique characters (plesiomorphies and/or

apomorphies) to characterize and group some snake taxa is difficult due to the limited morphological information that can be obtained due to their specialized and therefore simple body structure (Huang *et al*., 2009). Thus, molecular data appear to play a more important role in snake systematics. Over the last two decades, fragment(s) of mitochondrial DNA (mtDNA) and single copy of nuclear DNA have frequently been used as a versatile and effective marker to investigate the phylogenetic relationships among snakes (e. g., Kelly *et al*., 2003; Lawson *et al*., 2005; Burbrink and Lawson, 2007; Huang *et al*., 2009; Vidal *et al*., 2010; Pyron *et al*., 2011). For example, He *et al*. (2009) used cyt *b* and *c-mos*, and Huang *et al*. (2009) selected cyt *b* and ND4 to examine the systematic position of *Thermophis* within Caenophidia, and found that *Thermophis* appeared to be a member of the subfamily Dispadinae (then Xenodontinae; see Pyron *et al*., 2011). But their data failed to clarify the relationship between the New World xenodontines and *Thermophis*. Therefore, our goal of this paper is to provide sufficient data for a better understanding of the phylogenetic position of *Thermophis* among Colubroidea.

In this study, we sequenced 3 *Thermophis* and used 73 Caenophidian ND2 sequences from GenBank, as well as 12 henophiids as outgroups in a phylogenetic analysis with an attempt to clarify the phylogenetic relationship between Dipsadinae and *Thermophis*. The systematic nomenclature within Colubridae used in this paper was primarily based on the classification recommended by Lawson *et al*. (2005), which was derived from their phylogenetic analyses of cyt *b* and *c-mos* genes, as well as on the evidences obtained from previous work using morphological, immunological, and DNA sequence data. However, we simultaneously followed the recommendation by Pyron *et al*. (2011) in using the name Dipsadinae to refer to Xenodontinae (South American xenodontines + Central America dipsadines + North American relict snakes) due to their controversial status at the subfamilial level (e. g., Vidal *et al*., 2000; Lawson *et al*., 2005; Zaher *et al*., 2009). Much of the classification proposed by Lawson *et al*. (2005) has been adopted and corroborated by many contemporary workers in current literature (e. g., He *et al*., 2009; Huang *et al*., 2009; Ling *et al*., 2010).

2. Materials and Methods

2.1 Downloaded sequences and sampling information

We sampled 87 species (88 sequences) representing mostly Alethinophidia (primitive + advanced snakes; Table 1). Of these, 86 sequences were retrieved from GenBank, and 2 were unique sequences from 8 individuals of *Thermophis*: 6 *T. baileyi* from Yangbajain (HS200818-2; N: 30.0638; E: 90.4876; 4100 m.), Xizang (Tibet), China and 2 *T. zhaoermii* form Litang (HS200818-1; N: 29.6921, E: 100.3842; 3700m), Sichuan, China. The snakes collected for this study were humanely euthanized using lethal injection. The fresh liver tissues or tail tip biopsies were removed and immediately preserved in 95% ethanol for sequencing their mitochondrial ND2 gene. Specimens are deposited in the museums of Sichuan University and Sichuan Forestry Academy.

2.2 DNA extraction, amplification and sequencing
Eight individuals of *Thermophis* were collected during the summer of 2009. Three distal ends of their ventral scales were removed and immediately preserved in 95% ethanol for sequencing their mitochondrial ND2. We extracted total genomic DNA using the phenol/chloroform extraction procedure in Sambrook *et al*. (1989). Polymerase chain reaction (PCR) was used for amplification of mitochondrial ND2 with primer pairs L4437c, tRNA-trpR' (Huang *et al*., 2007). PCR was carried out under the following conditions: an initial cycle at 94° for 7 min followed by 40 cycles of 94° denaturation (40 s each), 56° annealing (30 s) and 72° extension (1 min) with a final extension at 72° for 7 min.

2.3 Phylogenetic inference
Newly obtained sequences were aligned with other retrieved sequences using Clustal X (Thomopson *et al*., 1997) and were translated into amino acids to check for the presence of stop codons within the sequences which might indicate the amplification of pseudogene(s). Nucleotide composition and the uncorrected *P*-distances were calculated in Mega 4.0 (Tamura *et al*., 2007).

Bayesian inference (BI) approaches were used to reconstruct phylogenetic bifurcating trees in the present study, using MrBayes V3.0 (Huelsenbeck and Ronquist, 2001). Modeltest 3.7 (Posada and Crandall, 1998) was used to find the model of nucleotide evolution using the Bayesian Information Criterion (BIC) for BI, with the scores formed by PAUP* V4b10 (Swofford, 2002). In the Bayesian analysis, a starting tree was obtained using the neighbor-joining algorithm. Posterior probabilities (PP) were obtained by Markov Chain Monte Carlo (MCMC) analysis with one cold chain and three heated chains. Bayesian tree estimation and support for the nodes was

Table 1 Information of the sequences retrieved from GenBank and sequenced in this study. Much of the classification is proposed by Lawson *et al.* (2005) but simultaneously follows the recommendation by Pyron *et al.* (2011) in using the name Dipsadinae to refer to Xenodontinae (South American xenodontines + Central America dipsadines + North American relict snakes).

Clade		Genus and species	Accession No.	Source
Colubridae				
	Colubrinae	*Ptyas korros*	AY487023	Nagy *et al.* (2004)
		Bogertophis subocularis	DQ902206	Burbrink and Lawson (Unpubl.)
		Pituophis deppei	FJ627848	Pyron and Burbrink (2009)
		Cemophora coccinea	DQ902249	Burbrink and Lawson (Unpubl.)
		Lampropeltis ruthveni	FJ627847	Pyron and Burbrink (2009)
		Stilosoma extenuatum	DQ902245	Burbrink and Lawson (Unpubl.)
		Pseudelaphe flavirufa	DQ902216	Burbrink and Lawson (Unpubl.)
		Arizona elegans	DQ902204	Burbrink and Lawson (Unpubl.)
		Pantherophis obsoletus	FJ627844	Pyron and Burbrink (2009)
		Rhinocheilus lecontei	FJ627838	Pyron and Burbrink (2009)
		Senticolis triaspis	DQ902237	Burbrink and Lawson (Unpubl.)
		Coelognathus radiatus	DQ902230	Burbrink and Lawson (Unpubl.)
		Oreophis porphyraceus	DQ902226	Burbrink and Lawson (Unpubl.)
		Elaphe quatuorlineata	AY487028	Nagy *et al.* (2004)
		Euprepiophis mandarinus	DQ902222	Burbrink and Lawson (Unpubl.)
		Dinodon semicarinatus	NC_001945	Kumazawa *et al.* (1998)
		Coronella girondica	AY487027	Nagy *et al.* (2004)
		Gonyosoma oxycephalum	DQ902241	Burbrink and Lawson (Unpubl.)
		Drymarchon corais	DQ902207	Burbrink and Lawson (Unpubl.)
		Salvadora mexicana	AY487036	Nagy *et al.* (2004)
		Masticophis flagellum piceus	AY487022	Nagy *et al.* (2004)
		Coluber constrictor mormon	AY487002	Nagy *et al.* (2004)
		Macroprotodon brevis	DQ902270	Busack and Lawson (2008)
		Hemerophis socotrae	AY487016	Nagy *et al.* (2004)
		Eirenis levantinus	AY487032	Nagy *et al.* (2004)
		Hierophis viridiflavus	AY487018	Nagy *et al.* (2004)
		Spalerosophis diadema	AY487020	Nagy *et al.* (2004)
		Hemorrhois ravergieri	AY487011	Nagy *et al.* (2004)
		Platyceps najadum	AY487009	Nagy *et al.* (2004)
		Oocatochus rufodorsatus	DQ902232	Burbrink and Lawson (Unpubl.)
		Orthriophis cantoris	DQ902246	Burbrink and Lawson (Unpubl.)
	Xenodermatinae	*Achalinus meiguensis*	NC_011576	Wang *et al.* (Unpubl.)
	Homalopsinae	*Enhydris plumbea*	NC_010200	Yan *et al.* (2008)
	Natricinae	*Amphiesma sauteri*	AF384824	Alfaro and Arnold (2001)
		Natrix tessellata	AY487776	Guicking *et al.* (2006)
		Virginia striatula	AF384852	Alfaro and Arnold (2001)
		Tropidoclonion lineatum	AF384850	Alfaro and Arnold (2001)
		Nerodia harteri	AF384854	Alfaro and Arnold (2001)
		Xenochrophis flavipunctatus	FJ416748	Hedges *et al.* (2009)
		Regina septemvittata	AF384836	Alfaro and Arnold (2001)
		Clonophis kirtlandii	AF384827	Alfaro and Arnold (2001)
		Thamnophis validus validus	EF417447	de Queiroz and Lawson (2008)
		Seminatrix pygeae	AF384839	Alfaro and Arnold (2001)
		Storeria occipitomaculata	AF384840	Alfaro and Arnold (2001)
	Dipsadinae	*Hypsiglena*	EU728580	Mulcahy and Macey (2009)
		Pseudoleptodeira latifasciata	EU728579	Mulcahy and Macey (2009)
		Imantodes cenchoa	EU728586	Mulcahy and Macey (2009)
		Leptodeira septentrionalis	EU728590	Mulcahy and Macey (2009)
		Sibon nebulatus	EU728583	Mulcahy and Macey (2009)
		Helicops angulatus	FJ416751	Hedges *et al.* (2009)
		Uromacer oxyrhynchus	FJ416754	Hedges *et al.* (2009)
		Darlingtonia haetiana	FJ416774	Hedges *et al.* (2009)
		Antillophis andreae	FJ416781	Hedges *et al.* (2009)
		Alsophis cantherigerus ruttyi	FJ416785	Hedges *et al.* (2009)

(Continued Table 1)

		Ialtris dorsalis	FJ416773	Hedges *et al.* (2009)
		Arrhyton funereum	FJ416777	Hedges *et al.* (2009)
		Hypsirhynchus ferox	FJ416780	Hedges *et al.* (2009)
		Heterodon platirhinos	FJ416750	Hedges *et al.* (2009)
		Farancia abacura	DQ902239	Burbrink and Lawson (Unpubl.)
	Dipsadinae (*incertae sedis*)			
		Thermophis baileyi	JF411072	This study
		Thermophis zhaoermii	JF411071	This study
		Thermophis zhaoermii	NC_012816	He *et al.* (2009.)
Acrochordidae		*Acrochordus granulatus*	NC_007400	Dong and Kumazawa (2005)
Viperidae				
	Viperinae	*Causus defilippi*	GU045452	Castoe *et al.* (2009a)
		Vipera aspis aspis	AY581266	Garrigues *et al.* (2004; Unpubl.)
		Daboia russellii	NC_011391	Chen and Fu (Unpubl.)
	Crotalinae	*Deinagkistrodon acutus*	DQ836185	Huang *et al.* (2007)
		Gloydius brevicaudus	DQ839399	Huang *et al.* (2007)
		Crotalus viridis viridis	AY704799	Wüster *et al.* (2005)
		Ovophis okinavensis	AB175670	Dong and Kumazawa (2005)
		Agkistrodon piscivorus	NC_009768	Jiang *et al.* (2007)
		Viridovipera stejnegeri stejnegeri	FJ752492	Lin *et al.* (Unpubl.)
Elapidae				
		Bungarus multicinctus	NC_011392	Chen and Fu (Unpubl.)
		Micrurus fulvius	GU045453	Castoe *et al.* (2009b)
		Naja naja	NC_010225	Yan *et al.* (2008)
		Ophiophagus hannah	NC_011394	Chen and Fu (Unpubl.)
Pythonidae				
		Python regius	NC_007399	Dong and Kumazawa (2005)
Boidae				
	Erycinae	*Charina trivirgata*	GQ200595	Douglas and Gower (2010)
	Boinae	*Eunectes notaeus*	AM236347	Douglas *et al.* (2006)
		Boa constrictor	NC_007398	Dong and Kumazawa (2005)
Xenopeltidae		*Xenopeltis unicolor*	NC_007402	Dong and Kumazawa (2005)
Cylindrophiidae		*Cylindrophis ruffus*	AY662538	Townsendb *et al.* (2004)
Uropeltidae		*Rhinophis philippious*	GQ200594	Douglas and Gower (2010)
Tropidophiidae		*Tropidophis haetianus*	FJ755181	Castoe *et al.* (2009b)
Aniliidae		*Anilius scytale*	GQ200593	Douglas and Gower (2010)
Leptotyphlopidae		*Rena humilis*	AB079597	Kumazawa and Nishida (1995)
Typhlopidae		*Typhlops reticulatus*	NC_010971	Castoe *et al.* (2008)
		Ramphotyphlops australis	AM236346	Douglas *et al.* (2006)

inferred using a Monte Carlo Markov chain model as implemented in MrBayes, with 10,000,000 generations after a "burn-in" of 3,000,000 generations. Sampling was performed every 1000 generations. We ran three additional analyses starting with random trees. Consensus of all the post-burn generations (3,600,000 generations resulting in 36,000 trees) was computed from all four runs. In addition, ML tree was derived using a RAxML BlackBox in the model of Maximum likelihood search (http:// phylobench.vital-it.ch/raxml-bb).

3. Result

3.1 Sequence characteristics The complete ND2 gene

sequences (1032 bp, i. e., 344 codons) of *T. baileyi* and *T. zhaoermii* were obtained in this study, both including the stop codon TAG located at the 3rd terminal end. An open reading frame was obtained for all ND2 sequences. The average nucleotide composition (%), numbers and percentages of variable (V) and PI sites for ND2 gene fragments of Caenophidia, Dipsadinae (excluding *Thermophis*, clustered in this subfamily, see below), and *Thermophis* used in the present study were listed in Table 2. The percentages of the four nucleotides were not significantly different across taxa groups, and showed strong bias against guanidine, typical for mitochondrial DNA (e. g., Lawson *et al.*, 2005; Huang *et al.*, 2009).

The uncorrected *P*-distance within Caenophidia,

Table 2 Average nucleotide composition (%), numbers and percentages (%) of variable (V) and parsimony informative (PI) sites for ND2 gene, based on the data used in the present study.

Taxa		T (U)	C	A	G	V (V/aligned sites)	PI (PI/aligned sites)
Caenophidia[*]	n = 73	22.8	30.5	37.8	8.8	754 (73.1)	663 (64.2)
Dipsadinae[*]	n = 15	22.7	31.1	37.3	8.9	525 (50.1)	319 (30.9)
Thermophis	n = 3	21.0	33.5	36.4	9.2	81 (7.9)	

n: Numbers of sequences of each group.
[*]: Excluding Thermophis.

Dipsadinae, and *T. baileyi vs. T. zhaoermii* ranged from 0.229, 0.192 to 0.078 (Table 3). The number of *T. zhaoermii vs. T. baileyi* (0.078) was slightly more than the minimum number of Caenophidia (0.072). This lends to support the validity of recognizing *T. zhaoermii* as a new taxon (Huang *et al*., 2009).

3.2 Topological results The topologies observed between the BI and ML trees were similar. Based on the ML and BI trees (Figure 1), *Acrochordus* and *Achalinus* are always subtended by the root node to the monophyletic Colubroidea. Viperidae formed a monophyletic group which was sister to a clade of Homalopsinae and Elapidae, as well as Natricinae, Dipsadinae and Colubrinae. The basic relationships of the commonly recognized families and/or subfamilies are similar to those of many earlier studies (e. g., Kelly *et al*., 2003; Burbrink and Lawson, 2007; Wiens *et al*., 2008; Huang *et al*., 2009).

Our data supported the monophylely of the dipsadines (99% in ML and 100% in BI) consisting of three subgroups: 1) "Clade Relicts": American relict snakes and *Thermophis* (weakly supported here; Pinou *et al*., 2004); 2) a strongly supported Central American group (92% in ML and 100% in BI); and 3) a strongly supported South American group (92% in ML and 100% in BI). South American and Central American clades huddled strongly and were supported by ML (74%) and BI (99%), and were sister to the root of "Clade Relicts".

4. Discussion

One of the major challenges to inferring large-scale phylogenies is the expense and time necessary to obtain tissue samples and comparable character sampling for hundreds of species and many genes (Pyron *et al*., 2011). Under limited conditions, we only sequenced the ND2 gene from two tissues (two species of *Thermophis*), retrieved 86 ND2 sequences from GenBank (74 Caenophidia and 12 Henophidia), and combined them to

Table 3 The uncorrected *P*-distances of ND2 gene, based on the data used in the present study.

Taxa		Uncorrected *P*-distance		
		Maximum	Minimum	Mean
Caenophidia[*]	n = 73	0.356	0.072	0.229
Dipsadinae[*#]	n = 15	0.255	0.123	0.192
T. zhaoermii vs. T. baileyi	n = 3			0.078

n: Numbers of sequences of each group.
[*]: Excluding Thermophis.
[#]: Including the sequences of Dipsadinae, Xenodontinae and North American relicts.

infer a large phylogenetic tree. The relatively high support in main nodes (Figure 1) demonstrates that ND2 is a good genetic marker for inferring large-scale trees of snakes.

Despite of the difference in nomenclature between the North, Middle, South American xenodontines and their subgroups by several researches (e. g., Pinou *et al*., 2004; Lawson *et al*., 2005; Hedges *et al*., 2009; Huang *et al*., 2009; Zaher *et al*., 2009; Vidal *et al*., 2010; Pyron *et al*., 2011), the relationships always cluster as: 1) xenodontines always consist of three parts, that is, Xenodontinae, Dipsadinae, and North American Relicts; 2) North American relicts served as outgroup of middle and south xenodontines; and 3) *Thermophis* was always placed together with (this study) or near (Pyron *et al*., 2011) the North American relicts. Huang *et al*. (2009) indicated that *Thermophis* is part of the subfamily Dipsadinae, one of the largest subfamilies of snakes with approximately 90 genera and more than 700 species, with all current members of the subfamily are restricted to the New World. In the present study, the closest relatives of *Thermophis* appear to be the North American Relicts (*Heterodon platirhinos*, *Farancia abacura*).

Some phylogenetic analyses discussed the origin, dispersal and faunal exchange of snake groups in the Holarctic Region (e. g., Vidal *et al*., 2000; Pinou *et al*., 2004; Burbrink and Lawson, 2007). Vidal *et al*. (2000)
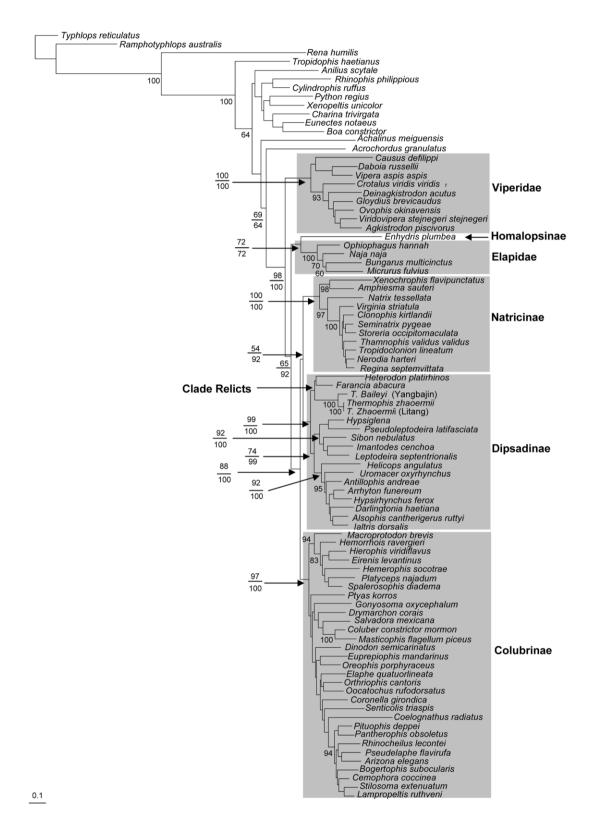
Figure 1 The ML tree based on ND2 sequences of 87 species of Alethinophidia. The values above the line are bootstrap supports of ML, and the values below are posterior probabilities (PP) of the 50% majority-rule consensus tree from BI (%). Likelihood settings from best-fit model (GTR + I + G) selected by Akaike and Bayeslan information criterion in Modeltest 3.7 (-lnL = 39597.2). Base = (0.4550, 0.3384, 0.0405), Nst = 6, Rmat = (0.1017, 2.9511, 0.1697, 0.1903, 1.7759), Rates = gamma, Shape = 0.5219, Pinvar = 0.1577.

inferred the phylogenetic relationships of the North, Middle and South American xenodontines from the sequence of portions of two mitochondrial genes (12S and 16S ribosomal RNA) in 85 species. They presented a hypothesis of an Asian–North American origin of dipsadines based on the monophyly of this group and their basal position with respect to North American taxa. In the present study, based on the analyses of the gene ND2, within the monophyletic clade of dipsadines (with supports of 99% in ML and 100% in BI), *Thermophis* clustered with the North American relicts to form a monophyletic subclade (despite with weak support), and rooted to Middle and South American xenodontines. This further suggested an Asian-North American origin of xenodontines. Similar topology (including Asian species of *Plagiopholis*, *Pseudoxenodon*, and/or *Thermophis*) was also revealed in the reconstructions of Lawson *et al*. (2005), Zaher *et al*. (2009), and Pyron *et al*. (2011). Extensive sampling (including *Plagiopholis*, *Pseudoxenodon*, *Thermophis*, and north, middle, south xenodontines) and same length genetic markers (including ND2) are needed in the future to obtain robust topologies with high support to further understand the history of this intercontinental group.

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